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A Single SNP Determines Blue or Brown Iris Pigmentation Successfully from Mock Evidence Samples for DNA Phenotyping

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ABSTRACT

The objective of this paper is to provide a simple assay for training biologists in the process of using single nucleotide polymorphisms, SNPs, for DNA phenotyping. A highly informative master switch SNP, rs12913832, was tested for the ability to successfully provide investigative information for the blue or brown eye color trait from several types of mock forensic samples. The TaqMan® SNP assay described here was found to be highly accurate for distinguishing the blue versus brown eye color iris phenotype. The SNP genotyping process described here used a variety of sample preparation methods and yielded a success rate of 96% for buccal swabs, 90% for hair roots and 50% for fingernail clippings.

Keywords: Forensic Biology Education; TaqMan® SNP Assay; Eye Color; Functional Genomics; SNP; Rs12913832; DNA Phenotyping; HERC2; Melanin Production; Forensic Science.

1.0. Introduction

With the increasing importance of forensic deoxyribonucleic acid (DNA) phenotyping, an assay for the use of biologically informative single nucleotide polymorphisms (SNPs) to resolve physical trait information was developed for training forensic biology students. This is a polymerase chain reaction (PCR) assay designed to target a single nucleotide base difference that results in two alleles, A or G, for the target locus. The rs12913832 locus is a master switch regulator to produce low or high melanin in iris melanocytes and results in blue or brown eye color, respectively, in humans as a scorable phenotype. The TaqMan® SNP assay has sequence-specific forward and reverse PCR primers to target the genetic location of interest and two minor groove binder (MGB) probes labeled with different fluorescent dyes that recognize either allele 1 (A) or allele 2 (G) of the target SNP. An instrument reads the fluorescence and creates a scorable allele discrimination plot to define the genetic variation.

1.1. Study Objectives

The following are the objectives of this study:

1. To define the genetic basis and use of allele variation in SNP assays.

2. To explain how blue and brown eye color phenotypes are correlated to SNP rs12913832.

3. To understand how to interpret an allele discrimination plot from a TaqMan® SNP assay.

4. To examine the success rate of the TaqMan® SNP assay used on different forms of mock biological evidence.

5. To understand the forensic relevance of the TaqMan® SNP assay for investigation and forensic DNA phenotyping for eye color for biological samples from a crime scene.

2.0. Literature Review

The single TaqMan® SNP assay was used to predict eye color using real-time PCR with allele discrimination scatter plot analysis [1-4]. This article presents the assay that establishes the difference between blue and brown eye color using the single human genome SNP, rs12913832 [5-6]. This assay aims to fill an important gap in science education, offering a clear genetic model to better understand how one nucleotide base difference can be applied to inform on forensic areas such as determining eye color from skeletal remains or profiling victims and perpetrator traits from biological hair, nails and other evidence that has been left behind at the crime scene [7-8]. The assay is simple to perform and requires approximately 1ng of purified DNA. Students can discover and classify their own eye color from a self-collected buccal swab or test forensically relevant samples such as hair and teeth to learn about forensic DNA phenotyping.

Iris pigmentation is determined by genetics and rs12913832 is a SNP in the HERC2 gene (chromosome 15) that regulates pigmentation of the iris during eye development (Figure 1). A SNP allele designation of A:A (homozygous allele1/allele1) or A:G (heterozygous allele1/allele2) determines brown eye color 80% of the time; a SNP allele designation of G:G (homozygous allele2/allele2) determines blue eye color 99% of the time. HERC2 stands for HECT domain and RLD2 and is one category of regulatory SNPs that affect phenotypes. Other important categories of SNPs include those that influence disease state, ancestry, drug efficacy and side effect risks. HERC2 is a master switch that controls another gene called OCA2 (named for a condition called oculocutaneous albinism type 2) that controls melanin production for the iris. The rs12913832 G:G allele condition reduces melanin production and results in blue eye color phenotype [9-13]. Thus, this single SNP is useful for the prediction of iris pigmentation from many types of forensically relevant biological samples. Although this is a single SNP assay using real-time PCR instrumentation, there are multi-SNP testing formats available that are based on sequencing methods [14-15].

Figure 1. Example of brown and blue iris pigmentation controlled by the rs12913832 SNP. Melanocytes produce eumelanin which is stored in specialized cells called melanocytes in brown eyes. Blue eye color is due to low melanin production and light scattering effects rather than blue pigment.

3.0. Materials and Methods

3.1. Sample Collection and Preparation of DNA

DNA collection was performed by students to collect a buccal swab from the interior cheek area for 30 seconds with a nylon swab. Swabs were air dried for 5 minutes and sealed in a 1.5mL Eppendorf microcentrifuge tube for DNA extraction. DNA extraction was performed by using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer protocol. The human component of the extracted DNA was verified by using the Quantifiler® Trio DNA Quantification kit (Thermo Fisher Scientific, Waltham, MA) per manufacturer protocol. The collected biological samples were approved by an Institutional Review Board (IRB) for human subject testing. Collected samples have been successfully used to generate SNP data 3-6 months post collection if stored at room temperature or at -20C. Blue and brown eye control samples should also be used with each rs12913832 (HERC2 gene associated, chromosome 15) TaqMan® SNP assay run for comparative purposes. A detailed close-up photograph of the student's eye is required for reference of perceived eye color and can be collected by cell phone camera. A similar process was used for hair root and nail samples except for an added initial 100% molecular biology grade ethanol (Thermo Fisher Scientific, Waltham, MA) rinse prior to DNA extraction. The hair root was collected from donor, cleansed with ethanol and extracted with a Quick-DNA Microprep Plus Kit (Zymo Research, Irvine, CA). The nail clippings were collected from donors with sterile nail clippers, rinsed in ethanol and DNA extracted with a BcMag™ One-Step Touch DNA Purification Kit (Bioclone, San Diego, CA).

3.2. The Taqman® SNP Genotyping Assay

The TaqMan® SNP Genotyping Assay (Thermo Fisher Scientific, Waltham, MA) was performed per manufacturer protocol using 1 nanogram of DNA template. The 40X custom primers (Assay ID C_30724404_10 for rs12913832, HERC2 gene) were ordered separately and were diluted 1:1 with sterile molecular biology grade water prior to use to a 20X concentration (Custom SNP Assay Mix). The master mix used in the assay was TaqMan™ Genotyping Master Mix (Thermo Fisher Scientific, Waltham, MA). The custom SNP assay was prepared in a MicroAmp™ Fast Optical 96-Well Reaction plate (Thermo Fisher Scientific, Waltham, MA). The assay recipe is: 10 uL TaqMan™ Genotyping Master Mix (2X), 1.0 uL Custom SNP Assay Mix (20X), 9.0 uL purified genomic DNA (1ng) for a final volume of 20uL per reaction. The PCR cycling parameters were set using the custom SNP assay module on a QuantStudio™ 5 PCR System (Thermo Fisher Scientific, Waltham, MA) to the following: polymerase enzyme activation at 95C for 10 min., followed by alternating 40 cycles each of denaturation at 95C for 15 sec. and annealing/extension at 80C for 1 min. The data was analyzed by a real-time PCR instrument and presented to the user as an allele discrimination scatter plot for scoring.

4.0. Result and Discussion

4.1. Allele Discrimination Plot

An example of an allele discrimination scatter plot is displayed by the real-time PCR instrumentation for the assay results (Figure 2). The allele combinations are color coded for homozygous allele1/allele1 (A:A, red), heterozygous allele1/allele2 (A:G, green) and homozygous allele2/allele2 (G:G, blue).

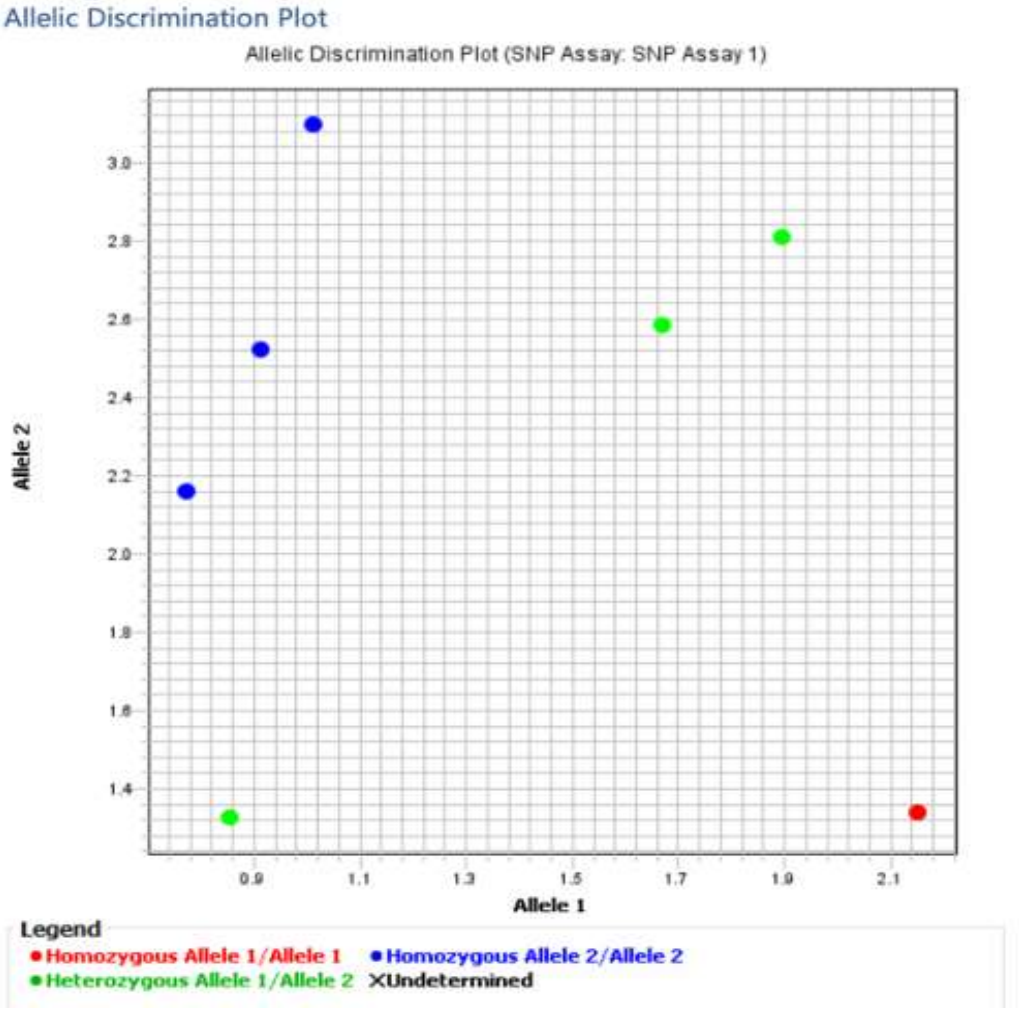

Figure 2. Example of an allele discrimination scatter plot of SNP data for rs12913832. The allele combinations are color coded for homozygous allele1/allele1 (A:A, red), heterozygous allele1/allele2 (A:G, green) and homozygous allele2/allele2 (G:G, blue).

4.2. Allele Scoring

Alleles are scored as allele 1/allele1 (homozygous condition), allele1/allele2 (heterozygous condition) or allele 2/allele2 (homozygous condition) based on positive control reference samples for blue and brown eye color and the sample genotypes are scored. HERC2 alone has a high accuracy for correct prediction of blue iris color; however, HERC2 in combination with the OCA2 rs1800407 SNP (GG on chromosome 15; for grey or blue eye phenotype) is an even better combination for accurate prediction since HERC2 regulates OCA2.

4.3. IrisPlex Software and SNPedia Classification Schemes

One of the challenges for students learning SNP analysis is to find accurate positive controls for genetic blue and brown eye color to aid in determining genotype from the scatter plot to clarify which allele combination correlates to the correct phenotype. Photographs to confirm eye color allele assignment from individuals with very dark brown or very light clear blue irises are useful as positive phenotype controls. IrisPlex eye color prediction software as a free download [10]; and descriptions of SNPs with the corresponding genotype and phenotype predictions available at SNPedia.com, are also useful as instructional resources [13].

The SNP assay has been applied to DNA extracted from buccal swabs, hair roots and fingernail clippings and allows for the empirical observation of student skill and ability to generate SNP data from a variety of mock evidence samples (Table 1).

This TaqMan® SNP assay has been popular with more than ten successful training sessions for forensic biology students over a period of three years. Interesting training issues have been identified for individuals with pigment chimerism (heterochromia) and for observed differences between genetic prediction and color perception as exceptions to the physical trait prediction accuracy. The SNP genotyping success rate varies based on sample type but ranges from 50 – 96%. The degradation index (DI) is a measure of the level of DNA fragmentation and a good predictor of SNP genotyping success rate. The formula for the calculation of the degradation index is the DNA concentration of the small autosomal target divided by the DNA concentration of the large autosomal target from the Quantifiler Trio data. A DI value of less than or equal to 1 reflects intact DNA. A DI value of 1 to 10 describes moderate degradation. DI values greater than 10 indicate significant DNA degradation. The greater the DI index, the greater the chance the genotyping step will fail.

Table 1. Percent genotyping success for the rs12913832 TaqMan® SNP Genotyping Assay.

Sample Type	DNA Extraction Kit	DNA Yield (ng)	Degradation Index (DI)	Sample Number	Percent Genotyping Success (%)
Buccal swab	PureLink™ Genomic DNA Mini Kit	0 – 200.2ng	0 – 1.62	N = 45	96%
Hair root	Quick-DNA Microprep Plus Kit	0.06 – 384ng	0.79 – 3.94	N = 10	90%
Nail clippings	BcMag™ One-Step Touch DNA Purification Kit	0 – 13.94ng	0.16 – 24.02	N = 12	50%

5.0. Conclusion and Future Recommendations

The use of the TaqMan® SNP Genotyping Assay for eye color prediction has been an invaluable training tool to educate forensic biologists in the process of using SNPs to make predictions about physical traits to aid in human identification [13]. In classroom discussion about the forensic DNA phenotyping process which begins with SNP profiling from crime scene evidence or skeletal human remains, this assay allows for the student to explore the single nucleotide base genetic variation used to classify a person by externally visible characteristics (EVC). When examining the blue eye color trait at the population level (Table 2), a biologist can determine the frequency of an expected blue eye phenotype in different founder populations. For example, since a G:G rs12913832 SNP allele combination would predict a blue eye phenotype with 99% accuracy, one can determine from Table 2 that this would not likely be an individual from the East Asian population since there is a 0% G observed allele frequency.

Table 2. Population allele frequencies for the rs12913832 SNP A and G alleles*

Population	**% G**	**% A**
Global	18	82
SAS (South Asian)	7	93
AFR (African)	3	97
EUR (European)	36	64
AMR (Amerindigenous)	20	80
EAS (East Asian)	0	100

*1000 Genomes Project (Thermo Fisher Scientific, Waltham, MA).

The research was developed to train forensic biologists in the implementation and execution of a simple master switch TaqMan® SNP Genotyping Assay for blue or brown eye color using a SNP designated as rs12913832. It has been extensively tested by forensic biology students with the majority achieving 50% or greater genotyping success with their biological samples. Successful samples included DNA extracts from buccal swabs, hair roots, and nail clippings; fingerprints from glass slides did not yield positive results due to a low yield (picogram quantities) of DNA from the extractions (data not shown). For this SNP assay, a significant quantity of DNA (1ng) is required for success and therefore, the quantitation step is critical to the performance of the SNP assay. As a TaqMan® SNP assay with a high accuracy prediction of blue versus brown eye color, this is a suitable choice for sorting candidate individuals in an investigation based on phenotypic eye color or for assessment of eye color from skeletal remains.

5.1. Future Recommendations

1. Future research can extend to the use of additional TaqMan® SNP Assays by changing forward and reverse PCR primers and MGB probes to target other informative areas of the human genome.

2. The study populations and sizes can be increased to include the effect of ancestry on the success rate of the TaqMan® SNP Assay.

3. DNA collection and extraction methods can be optimized for DNA from fingerprints to determine if this TaqMan® SNP Assay could be useful for fingerprint samples in the future.

4. An analysis of the phenotypic variation observed from the reference eye color photographs can define the shades of blue and brown eye color that are predicted most accurately with this TaqMan® SNP Assay.

5. Reference populations can be created in the classroom to compare the observed frequency of blue and brown eye color phenotypes with the expected frequencies shown in Table 2.

Declarations

Source of Funding

The research was solely funded by University of New Haven.

Competing Interests Statement

The authors declare no competing financial, professional, or personal interests.

Consent for Publication

The authors declare that they consented to the publication of this study.

Authors' Contributions

All the authors took part in literature review, analysis and manuscript writing equally.

Availability of Data and Materials

Supplementary information is available from the authors upon reasonable request.

Institutional Review Board Statement

Informed consent was required for human subject testing and was approved by the University of New Haven IRB Committee for this research.

Ethical Approval

All research was performed in an ethical manner.

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Declaration of Artificial Intelligence

No artificial intelligence (AI) was used in this study or for the publication of this manuscript.

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